The University of Chicago

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I. GROWTH OF CLOSTRIDIUM BOTULINUM ON CASEIN HYDROLYSATE AND ON HYDROLYSATE PREPARATIONS

II. GROWTH OF CLOSTRIDIUM BOTULINUM ON SYNTHETIC MEDIUMS

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY
1932

By
WILLIAM BURROWS

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GROWTH OF CLOSTRIDIUM BOTULINUM ON CASEIN HYDROLYSATE AND ON HYDROLYSATE PREPARATIONS

WILLIAM BURROWS
CHICAGO

The relationship of the growth requirements of the obligate anaerobes to individual amino-acids has never been investigated, with the exception of a small amount of work on the relationship to cystine. The investigation by Hosoya and Kishino [1] indicated the importance of the sulphydryl group in the metabolism of obligate anaerobes; they found that cultures of Cl. botulinum and Cl. tetani would not grow on tryptic digests of gelatin except when the basal source of nitrogen was supplemented with cystine or with sodium sulphide. Their work further showed that elementary sulphur, various sulphates, taurine and sodium taurocholate would not serve as sources of sulphur.

Quastel and Stephenson [2] found that Cl. sporogenes, while developing readily on acid and tryptic digests of casein, was unable to grow on similar digests of gelatin. They conducted experiments which, they considered, showed that the inhibiting factor in the gelatin preparations was the lack of reduced compounds of sulphur, and found that the addition of reduced glutathione, cysteine and thioglycollic acid permitted growth. The authors expressed the belief that the prime function of such compounds is that of a donator of hydrogen in the production of rH values within the limits necessary to the growth of obligate anaerobes.

These investigations are open to criticism on two major points: First, the addition of dry crystalline compounds to bacterial cultures is fraught with considerable danger of contamination. In the second place, no precautions were taken to control the identity of the growing organisms.

From the Department of Hygiene and Bacteriology of the University of Chicago.

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1. Scient. Rep. Govt. Inst. Infect. Dis., Tokyo **4**:123, 1925.
2. Biochem. J. **20**:1126, 1926.

The present investigation was undertaken in an effort to determine the relationship of as many individual amino-acids as possible to the growth of various strains of Cl. botulinum, types A and B.

MATERIALS AND METHODS

An acid hydrolysate of casein was prepared according to the method of Berg and Rose.[3] The commercial grade of protein was hydrolyzed with strong sulphuric acid, the sulphate exactly removed with barium, the solution concentrated *in vacuo,* dried on a water bath and in a hot air oven, and ground to a fine powder. Arginine was removed from two portions of this hydrolysate as the flavianate by the method of Bunney and Rose.[4] One of these portions and another portion of the hydrolysate were treated according to the method of Kingston and Schryver [5] to remove the dicarboxylic acids, aspartic acid, hydroxyglutamic acid and glutamic acid. These acids are removed by the precipitation of their barium salts from alcohol. Other procedures, such as the removal of histidine as the silver salt, etc., were not considered suitable, because of the incidental removal of other amino-acids in unknown quantities. It was felt that, as far as possible, the amino-acid content of the material after treatment should be fairly accurately known.

There are two possible objections to the use of the material resulting from treatment with flavianic acid as the nitrogenous base of a medium. First, traces of flavianic acid remaining may be toxic. This is not probable in view of its atoxicity for higher animals, and experiments indicated that the traces of the dye present have no deleterious effect on the growth of Cl. botulinum. Second, in the event of an incomplete removal of hydrochloric acid from the flavianic acid, there might possibly be present a sufficient amount to affect the growth of the organisms. However, a simple calculation indicates that when the material is made up in 1 per cent solution for use as a medium, the final concentration of chloride from this source is negligible. Cl. botulinum tolerates from 7 to 8 per cent sodium chloride.

The constituent tryptophan of casein is destroyed during the course of acid hydrolysis and supposedly plays an active part in the formation of insoluble humin.

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3. J. Biol. Chem. **82**:479, 1929.
4. J. Biol. Chem. **76**:521, 1928.
5. Biochem. J. **18**:1070, 1924.

A portion of the hydrolysate was treated with norite (a vegetable charcoal) for the removal of soluble humin by adsorbtion. It was necessary to adsorb twice before the solution of hydrolysate came through the filter water clear.

The light brown powder, referred to as the casein hydrolysate, contains the constituent amino-acids of the original casein, with the exception of tryptophan, small amounts of ammonia, soluble humin, anhydrides and condensation products. No attempt was made to suppress oxidation during the course of hydrolysis by the use of stannous chloride or titanious chloride as described by Sullivan.[6] The composition of casein, based on the best determinations of various chemists, is given by Mendel and others [7] as:

	Per Cent
Glycine	0.45
Alanine	1.85
Amino-butyric acid	None
Valine	7.93
Isoleucine	None
Leucine	9.70
Serine	0.50
Phenylalanine	3.88
Tyrosine	6.50
Proline	7.63
Oxyproline	0.23
Aspartic acid	4.10
Glutamic acid	21.77
Oxyglutamic acid	10.50
Histidine	2.84
Arginine	3.81
Lysine	7.62
Cystine	0.50
Tryptophan	2.20
Ammonia	1.61

Discriminate use of the procedures mentioned and the utilization of each of the preparations resulting from these procedures as a source of nitrogen in culture mediums make possible a number of mediums differing from one another only in their amino-acid content. As the

6. Pub. Health Rep., 1929, supp. 80.

7. Mendel: Nutrition, The Chemistry of Life, New Haven, Conn., Yale University Press, 1923. Hawk and Bergeim: Practical Physiological Chemistry, ed. 9, Philadelphia, P. Blakiston's Son & Co., 1927. Bodansky: Introduction to Physiological Chemistry, New York, John Wiley & Sons, 1927.

procedures have been applied here, the following combinations are possible: (1) casein hydrolysate; (2) hydrolysate with tryptophan added; (3) hydrolysate without arginine; (4) hydrolysate without arginine, with tryptophan added; (5) hydrolysate without dicarboxylic acids; (6) hydrolysate without dicarboxylic acids, with tryptophan added; (7) hydrolysate without dicarboxylic acids and arginine; (8) hydrolysate without dicarboxylic acids and arginine, with tryptophan added; (9) hydrolysate with humin removed, and (10) hydrolysate with humin removed and tryptophan added.

Mediums were made up from these preparations in the following manner:

A 1 per cent solution of the nitrogenous material was made in distilled water; 0.1 per cent of dextrose was added, the medium adjusted to p_H 7.4 to 7.6 with sodium hydroxide, and the material distributed in test tubes and covered with a mixture of petrolatum and paraffin. The mediums were sterilized in the autoclave at 20 pounds (9 Kg.) pressure for thirty minutes. The finished mediums were light brown and perfectly clear. In a few cases, a dark, granular precipitate, small in amount, appeared in the bottom of the test tubes after sterilization.

A number of preliminary experiments were carried out to determine the best technic to use, particularly in regard to the estimation of growth. It was soon apparent that accurate estimation of growth is particularly difficult in the case of the obligate anaerobes. Attempts were made to make use of the disappearance of dextrose and the appearance of ammonia, but these proved unsatisfactory, for the relative values secured not only failed to correspond with growth as indicated by turbidity, but were subject to wide variations in duplicate series of cultures. This was also true of estimations of aminonitrogen by the Sorensen technic. The hemocytometer count was finally adopted as the most exact and significant criterion of growth, even though it runs deceptively high. In conjunction with hemocytometer counts, attempts were made to estimate viable organisms by means of quantitative dilutions in beef infusion dextrose agar shake cultures. The counts so obtained varied so much among themselves that the procedure was useless for quantitative work. The presence of toxin was determined by the injection of 0.1 cc. of the culture into white mice weighing from 20 to 25 Gm.

When toxin could be demonstrated in growing cultures, the identity of the growing organisms was controlled by injection of the material into antitoxin-protected mice. If toxin was not found, it was necessary to resort to dilution of the culture sufficient to rule out the survival of

inoculated organisms, followed by culture in deep tubes of beef infusion agar. From this shake culture two colonies were picked and cultured in beef heart medium, and the specific toxigenicity of these subcultures was demonstrated by animal inoculation. These procedures, with the inoculation of agar slants at intervals, were judged sufficient to rule out contamination. These control measures gave satisfactory results throughout the investigation.

The cultures were:

Culture 1—type B—isolated in this laboratory from canned onions [8]
Culture 2—type B—isolated in this laboratory during the course of an investigation of the Westfield epidemic [9]
Culture 3—type A—recovered from canned spinach [10]
Culture 4—type B—Hygienic Laboratory no. 372
Culture 6—type A—American Type Culture no. 442
Culture 7—type A—American Type Culture no. 687

These cultures were tested for toxigenicity and type before use. Suspensions of spores were prepared from cultures in beef infusion dextrose broth from 4 to 6 weeks old. Smears from these cultures showed that they consisted almost entirely of spores and a small amount of cellular débris. The spores were washed five times by centrifugation from saline solution, suspended in saline solution, and standardized by hemocytometer count. The standard inoculum was 0.1 cc. of such a suspension, standardized to contain 1,000,000 spores per cubic centimeter. These suspensions were heated to from 80 to 90 C. for fifteen minutes to destroy any remaining traces of toxin and possible vegetative contaminants. After heating, atoxicity was checked by animal inoculation, sterility as to aerobic contaminants by culture on agar slants, and viability of spores by anaerobic cultivation and by the specific toxigenicity of such cultures. Anaerobic contaminants were not ruled out, but the precautions taken to insure the identity of growing cultures cover this point.

Cultures in hydrolysate mediums were observed three times at six day intervals. At each observation, in addition to the control procedures noted, record was made of turbidity, gas formation, hemocytometer count and presence or absence of toxin, except when otherwise noted. Incubation was at 37 C.

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8. Koser and Reiter: J. Prev. Med. **3**:499, 1929.
9. Cary and Koser: J. Prev. Med. **3**:317, 1929.
10. Koser, Edmondson and Giltner: J. A. M. A. **77**:1250, 1919.

EXPERIMENTS

Experiment 1.—A medium was prepared of the acid hydrolysate of casein, and tryptophan was added to replace that destroyed in the hydrolysis. Cultures 1 and 3 were used as representative type A and B cultures. The results are recorded in table 1.

Since these two cultures grew satisfactorily on this medium, another experiment was carried out to ascertain whether or not the other cultures on hand would grow on such a medium.

Experiment 2.—A medium containing the acid hydrolysate of casein with added tryptophan as a source of nitrogen was prepared. Cultures

TABLE 1.—*Growth of Cl. Botulinum on Casein Hydrolysate Tryptophan Medium*

Culture	6 Days				12 Days				18 Days			
	Turbidity	Gas	Count*	Toxin†	Turbidity	Gas	Count	Toxin	Turbidity	Gas	Count	Toxin
1 a	+++	+	36.5	12	+++	+	42.9	18	+++	+	40.1	
b	++++	+	52.1	12	++++	+	58.3	36	++++	+	51.3	72
c	++	+	23.6	24	+++	+	32.4	24	++++	+	45.1	36
d	++++	+	56.3	36	++++	+	65.7	24	++++	+	64.8	
e	+++	+	38.8	18	++++	+	43.7	36	++++	+	45.0	48
f	+++	+	31.0		++++	+	47.3		++++	+	41.8	
3 a	++++	+	48.7	12	++++	+	56.8	12	++++	+	55.4	24
b	++++	+	53.9	18	++++	+	61.5	18	++++	+	65.4	24
c	+++	+	37.2	24	++++	+	44.0	18	++++	+	45.2	48
d	++	+	22.3		+++	+	31.6	72	+++	+	38.0	
e	++++	+	59.8	24	++++	+	65.1	18	++++	+	74.6	
f	++++	+	49.0	72	++++	+	42.0	72	++++	+	43.8	

* The count is recorded in millions.
† The figures represent the number of hours elapsing between injection of the culture and death.

1, 2, 3, 4, 6 an 7 were used. Ten tubes of the medium were used for each culture. All showed marked turbidity and gas formation by the fifth day of incubation.

Since it was evident that these cultures of Cl. botulinum grow easily and well in a medium in which an acid hydrolysate of casein supplemented by tryptophan serves as a source of nitrogen, an experiment utilizing the hydrolysate alone was indicated.

Experiment 3.—Two mediums were prepared as in experiment 1, except that tryptophan was not added in one. Cultures 1 and 3 were used. The progress of growth in the medium containing tryptophan is given in table 2. Signs of growth could not be detected in the medium without tryptophan even though the period of observation was extended to one month.

In view of this interesting observation on the relationship of tryptophan to the growth of these cultures, a more extensive experiment including all the cultures on hand was carried out.

Experiment 4.—Two mediums were prepared as in experiment 3. Thirty tubes of each medium were used for each culture, and all the cultures on hand were used. By the twelfth day of incubation, all the tubes of medium containing tryptophan showed strong growth and gas formation, while the tubes of medium to which tryptophan had not been added showed no growth whatever. At the end of a month of

TABLE 2.—*Growth of Cl. Botulinum Brought About by Addition of Tryptophan to Hydrolysate Medium*

Culture	6 Days				12 Days				18 Days			
	Turbidity	Gas	Count*	Toxin†	Turbidity	Gas	Count	Toxin	Turbidity	Gas	Count	Toxin
1 a	+++	+	43.1	36	+++	+	40.3	48	+++	+	41.0	72
b	++++	+	48.8	12	++++	+	53.4	18	++++	+	50.6	24
c	+++	+	32.1		+++	+	38.5		+++	+	41.1	
d	++	+	18.2		+++	+	38.0	36	+++	+	32.3	
e	++	+	28.0	24	+++	+	35.4		+++	+	34.4	72
f	+++	+	44.4	18	++++	+	51.3	18	++++	+	50.0	36
3 a	++++	+	45.2	12	++++	+	49.7	12	++++	+	55.3	18
b	+++	+	37.4	18	++++	+	43.9	18	++++	+	42.6	24
c	++++	+	41.0	18	++++	+	43.8	12	++++	+	47.9	12
d	++++	+	54.6	24	++++	+	55.0	24	++++	+	52.1	18
e	++	+	22.5		++++	+	43.8	36	++++	+	42.5	
f	++++	+	41.3	12	++++	+	44.3	12	++++	+	39.0	12

* The count is recorded in millions.

† The figures represent the number of hours elapsing between injection of the culture and death.

incubation, the sediment at the bottom of ten tubes showing no growth was examined microscopically, with negative results. These cultures were kept for a period of six months, during which no new growth appeared. Two months later this experiment was repeated, with identical results.

Since tryptophan seemed to be so intimately concerned with the growth of these cultures of Cl. botulinum, the hydrolysate preparations from which other amino-acids had been removed were not used without tryptophan.

Experiment 5 was undertaken to determine the effect of the amino-acids arginine, aspartic acid, glutamic acid and oxyglutamic acid on the growth of Cl. botulinum.

Experiment 5.—Mediums were prepared in which the sources of nitrogen were (1) the hydrolysate from which arginine had been removed, (2) the hydrolysate from which the dicarboxylic acids had been removed, (3) the hydrolysate from which both arginine and the dicarboxylic acids had been removed, and (4) the hydrolysate from which the color had been removed by adsorption on ivorite. Two tubes of each medium were used for each culture. All the cultures were used. The data on the growth of the cultures in medium 1 are given in table 3. No signs of growth were apparent in

TABLE 3.—*Growth of Cl. Botulinum on Casein Hydrolysate Tryptophan Medium from Which Arginine Had Been Removed*

Culture	6 Days				12 Days				18 Days			
	Turbidity	Gas	Count*	Toxin†	Turbidity	Gas	Count	Toxin	Turbidity	Gas	Count	Toxin
1 a	.....	..	....	..	+++	+	46.5	72	++++	+	52.1	36
b	.....	..	....	..	+++	+	33.5		+++	+	38.9	24
2 a	.....	..	....	..	+++	+	44.2		+++	+	41.7	
b	.....	..	....	..	+++	+	46.8		++++	+	47.6	
3 a	++	..	28.2		++++	+	50.0	72	++++	+	48.3	24
b	.....	..	....	..	+++	+	44.9		++++	+	52.7	
4 a	.....	..	....	..	+++		34.4	36	+++	+	37.8	36
b	.....	..	....	..	++++		45.6		++++	+	51.3	18
6 a	.....	..	....	..	+++	+	45.7	24	++++	+	36.2	
b	.....	..	....	..	.....	..	....	..	.....	..	....	..
7 a	.....	..	....	..	++++		42.1		++++	+	48.5	24
b	.....	..	....	..	++++		45.2		++++	+	52.0	

* The count is recorded in millions.
† The figures represent the number of hours elapsing between injection of the culture and death.

the other three mediums, even though the period of observation was extended to one month. Later this experiment was repeated with the use of ten tubes of each medium for each culture, and these results were confirmed. It may be noted parenthetically that when, in a similar series of experiments, 0.01 cc. of a six day beef heart culture of the organisms was used as an inoculum in place of the spore suspension, growth ensued in medium 2 also.

The significance of tryptophan in the growth of Cl. botulinum as indicated by these experiments was of great interest. A further experiment was carried out in which an attempt was made to substitute structurally similar compounds for tryptophan in the untreated hydrolysate medium. Tryptophan, it will be remembered, is α amino β indole

propionic acid. The only compounds available were indole, skatole and indole 3-n-propionic acid. Because of the volatile nature of indole and skatole, sterilization of mediums containing these compounds by heat was contraindicated. In consequence, the mediums were sterilized by filtration through a Seitz-Werke positive pressure filter. However, a control medium containing tryptophan and prepared in this manner showed such poor growth that it was found necessary to prepare a medium without tryptophan as in experiment 3 and to sterilize solutions of the indole compounds by filtration and add these materials to the otherwise complete medium.

Experiment 6.—Mediums containing casein hydrolysate supplemented by the indole compounds tryptophan, indole, skatole and indole 3-n-propionic acid were prepared by the method given. Cultures 1 and 3 were used, and twelve tubes of each medium were used for each culture. Growth in the control medium containing tryptophan was excellent, but even at the end of a month no signs of growth were apparent in the mediums containing indole, skatole and indole 3-n-propionic acid.

Experiment 7.—Further experiments were conducted in which the hydrolysate medium without tryptophan, described in experiment 3, was used with varying amounts of tryptophan. The results were extremely difficult to tabulate, but it was found that a definite quantitative relationship appears to exist between the amount of tryptophan present and the profuseness of growth of cultures 1 and 3.

COMMENT

These observations show that the cultures of Cl. botulinum used in this investigation are able to grow on a medium in which the only source of nitrogen is an acid hydrolysate of casein with tryptophan added. The fact that the organisms grow and produce toxin on a medium of this sort indicates that whole proteins and their higher split products are not necessary for either growth or production of toxin. This does not appear to support the hypothesis of Stark, Sherman and Stark[11] that botulinum toxin is formed by the action of extracellular enzymes on protein materials of the medium, unless the reservation is made that the protein material in question may be that of disintegrated bacilli.

11. J. Infect. Dis. **43**:565, 1928.

Experiment 5 clearly indicates that arginine has no particular relationship to the development of the organisms. The same experiment shows the atoxicity of traces of flavianic acid.

The relationship between the growth of the organisms and the dicarboxylic acids is not so clear. Their removal is not quantitative, as is that of arginine, and it would appear that perhaps more than small amounts are necessary for the growth of the organisms. However, this conclusion may not be drawn, even tentatively, for there may be adsorption of necessary nutritive materials on one or more of the precipitates encountered during the course of removal.

Lack of growth on the hydrolysate medium that had been treated with norite, and the paucity of growth on the medium sterilized by filtration, may be accounted for by the hypothesis that necessary nutrient materials are removed by adsorption. It was noted that the filtrate from the Seitz filter was much lighter in color than the solution before filtration. It is possible that soluble humin itself is this nutrient material, but in a complex mixture such as a protein hydrolysate there is a multiplicity of substances that might be so adsorbed. If the adsorption hypothesis is correct, the identity of the substance or substances removal of which renders the casein hydrolysate unable to support the growth of Cl. botulinum can be nothing more than a matter of speculation at the present writing.

The most suggestive fact brought to light is the failure, in these experiments, of these cultures to grow without tryptophan. The observation that a medium that does not support the growth of these organisms supports their growth when modified only by the addition of pure tryptophan may be explained in several different ways. Tryptophan may exert a sensitizing action or may have a stimulating effect on the inoculated organisms which results in their proliferation. This is unlikely in view of the quantitative relationship between growth and the amount of tryptophan that was observed. There is also a possibility that tryptophan, as prepared, may be contaminated with vitamin-like substances. It seems probable that substances of this sort, if present in casein, would be destroyed by long continued boiling with strong sulphuric acid. However, it will be recalled that optically active tryptophan is prepared from enzymatic digests and might conceivably contain these hypothetic factors. This growth-stimulating effect of tryptophan may also be explained by the hypothesis that certain compounds or structures are "essential" for the growth of some of the more fastidious bacteria in a way analogous to that in which certain amino-

acids are "essential" to higher animal life. It will be remembered that the amino-acids lysine, tryptophan, cystine and histidine are essential to the growth of white rats, and the inclusion of tryptophan in this group is of particular interest in view of the experiments described in this paper. The fact that indole, skatole and indole 3-n-propionic acid could not substitute for tryptophan in the case of the two cultures used lends support to this hypothesis. The observation of Logie [12] that a large number of gram-negative bacilli, including Friedländer's bacillus and members of the colon-typhoid-dysentery group, not only did not need tryptophan, but actually formed it, is of interest. Tryptophan, like other amino-acids, is a constituent of all complete proteins, and it is probable that most bacterial proteins contain tryptophan. Consequently, if it must be supplied before growth can take place, it would seem that the organisms in question cannot synthesize it from any of the materials present in an acid hydrolysate of casein. It is evident that indole, skatole and indole 3-n-propionic acid are not precursors of tryptophan in this particular case. Whether or not this is true of all strains of Cl. botulinum is problematic. A culture on hand, supposedly Cl. botulinum, did not have this property, but since no toxin, specific or nonspecific, could be demonstrated, it was concluded that probably the culture was not Cl. botulinum.

CONCLUSIONS

Arginine was not an important factor in the growth of Cl. botulinum.

Traces of flavianic acid were not detrimental to the growth of Cl. botulinum.

Adsorption of solutions of casein hydrolysate on norite or on Seitz-Werke filter pads rendered them unfit to support the growth of Cl. botulinum even when supplemented by the addition of tryptophan.

Tryptophan was necessary to the growth of these strains of Cl. botulinum in a medium in which the source of nitrogen was an acid hydrolysate of casein.

No cultural differences were detected, on these mediums, between type A and type B cultures of Cl. botulinum.

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12. J. Path. & Bact. **23**:224, 1920.

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GROWTH OF CLOSTRIDIUM BOTULINUM ON SYNTHETIC MEDIUMS

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The relationships of the amino-acids arginine, aspartic acid, glutamic acid, oxyglutamic acid and tryptophan to the growth of some strains of Clostridium botulinum were reported in a previous paper.[1] The apparent intimate relationship existing between the presence of tryptophan and the growth of the organisms was suggestive and resulted in the development of a hypothesis, the essentials of which were formulated at that time.

Any species of bacterium may, theoretically, be grown in vitro provided the proper conditions are fulfilled. Such conditions include partial pressures of oxygen and carbon dioxide, temperature, hydrogen ion concentration, partial or complete anaerobiosis and oxidation-reduction potential, the chemical composition of the substrate on which it is proposed that the organisms be grown, and, perhaps, others not known. The most important single factor, in some cases at least, is the chemical composition of the substrate. The question arises, in the case of the more fastidious bacteria: Just what constitutes the proper substrate?

Nothing further should be necessary, from an empiric point of view, if sources of carbon and nitrogen are supplied in such forms that they are not toxic to the bacterial cell and yet are susceptible to attack by the bacterial synthetic and catabolic mechanisms. Numerous failures to grow some bacteria on synthetic mediums constitute ample evidence that this does not always hold true. A synthetic medium is defined as one in which the nutritive materials supplied are definite chemical compounds of known structure. These failures may be explained by the postulation of limits in the synthetic powers of the organisms in question. It is well known that more highly organized forms of life are limited in this respect. The assumption that this is the case among some of the bacteria is, perhaps, justified in spite of the fact that there is no morphologic indication to suggest it.

If it is true that some bacteria require not only food materials but also particular kinds of food materials, one would expect that this

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1. J. Infect. Dis. **51**:298, 1932.

peculiarity would be manifest in the more complex nitrogen metabolism rather than in the relatively simpler process of carbohydrate oxidation. It will be recalled that an analogous situation exists in the metabolism of higher animals. Carbohydrate in many forms is digested, absorbed and oxidized. In the metabolism of nitrogenous compounds there comes into play the phenomenon of "essential" amino-acids.

All the twenty amino-acids known to occur in proteins are essential to living protoplasm because from them it must synthesize its own particular and specific proteins. Some amino-acids, however, cannot be synthesized in vivo from materials usually [2] occurring in the diet, and therefore must be supplied as such either in the free state or combined in the form of proteins or their split products. Most authorities agree, at present, that at least four amino-acids occupy this position in regard to the metabolism of higher animals, these four being histidine, tryptophan, lysine and cystine.

If an analogous situation exists in the bacterial world, it should be possible to grow any species of bacterium on synthetic mediums provided that the proper amino-acids are supplied. If this should prove to be the case, previous negative results might be accounted for on the ground that the proper amino-acids were not supplied in the proper amounts.

On the assumption, as a working hypothesis, that there are "essential" amino-acids for some bacteria, at least, and Cl. botulinum in this particular case, it has been possible to prepare a synthetic medium, relatively simple in composition, which will support the growth of these organisms. At the same time evidence has been secured which apparently supports this hypothesis.

MATERIALS AND METHODS

The methods used in this work were essentially those outlined in a previous paper.[1] The strains of Cl. botulinum used were the following:

Strain 1—isolated in this laboratory from canned onions.[3]

Strain 2—isolated in this laboratory during the course of an investigation of the Westfield epidemic.[4]

Strain 3—recovered from canned spinach.[5]

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2. The word "usually" is used here, for Cox and Rose (J. Biol. Chem. **67**:3, 1926) and Norvello, Harrow and Sherwin (ibid. **67**:54, 1926) have been able to replace histidine in the diet of white rats with imidazole lactic acid, and Sullivan (Pub. Health Rep. **46**:1294, 1931) has been able to substitute dithio-ethylamine for cystine in the diet of white rats.

3. Koser and Reiter: J. Prev. Med. **3**:499, 1929.

4. Cary and Koser: J. Prev. Med. **3**:317, 1929.

5. Koser, Edmondson and Giltner: J. A. M. A. **77**:1250, 1919.

These strains were tested for toxigenicity and type before use. Spore suspensions were prepared from cultures in beef infusion dextrose broth from 4 to 6 weeks old. The spores were washed five times by centrifugation from saline solution, suspended in saline solution, and standardized by hemocytometer count. The standard inoculum was 0.1 cc. of such a suspension standardized to contain 1,000,000 spores per cubic centimeter. The suspensions were controlled for atoxicity by inoculation in mice, for sterility as regards aerobic contamination by culture on agar slants, and for viability of spores by anaerobic cultivation and tests for specific toxigenicity.

In some cases washed vegetative cells were used for inoculation instead of spores. These suspensions were prepared from eighteen hour cultures in veal infusion dextrose broth to which 0.1 cc. of a sterile 1 per cent solution of cysteine hydrochloride had been added just previous to inoculation. It had been noted previously that such an addition stimulates the organisms to rapid and profuse growth. These vegetative forms were removed by centrifugation and washed five times by centrifugation from saline solution to which a small amount of cysteine hydrochloride had been added. After washing, the cells were suspended in saline solution containing a small amount of cysteine hydrochloride, and the suspension was standardized by hemocytometer count to contain 1,000,000 cells per cubic centimeter; 0.1 cc. of such a suspension was used for inoculation. The toxicity of these suspensions and their sterility as regards aerobic contaminants and their viability were controlled in the same manner as in the case of the spore suspensions. Needless to say, the preparation of this sort of inoculum must take place just previous to use.

Sterile solutions of cysteine hydrochloride were prepared by dissolving the cysteine hydrochloride in distilled water saturated with hydrogen and sterilizing by filtration through a positive pressure Seitz-Werke filter, hydrogen being used to supply a pressure of 100 mm. of mercury. The receiving flask was so arranged that a small stream of hydrogen bubbled constantly through the filtrate. Such solutions are fairly stable for short periods of time when stored in an atmosphere of hydrogen.

The amino-acids used were those supplied by the Eastman Kodak Company, with the exception of lysine, oxyproline and some of the proline. Owing to the fact that this company no longer manufactures lysine, it was necessary to prepare it by the electrical transport method of Cox, King and Berg.[6] The first proline used was that supplied by the Eastman Kodak Company, but it was brown and amorphous. Later on, however, a more satisfactory product was secured from this

6. J. Biol. Chem. **81**:755, 1929.

source. Proline was prepared by Rose's [7] modification of Towne's [8] method—the separation of proline from hydrolyzed gelatin as the copper salt, followed by purification as the picrate. Proline was also prepared from Dakin's [9] butyl alcohol extract of both casein and gelatin hydrolysates. The purification of this product was difficult and unsatisfactory. However, a slight modification in Dakin's procedure was introduced which, when followed by repeated recrystallization and purification as the picrate, yielded a fairly satisfactory product.

Hydrolyzed protein, gelatin or casein was extracted with butyl alcohol in a continuous extractor of the Kütscher type for thirty-six hours. At the end of that time, the extract was evaporated to dryness in vacuo and the residue extracted with absolute alcohol. The alcoholic extract was allowed to stand for twenty-four hours; it was then filtered, the filtrate evaporated to dryness in vacuo and the residue dissolved in distilled water. This solution was shaken with ether and concentrated to a thin syrup under reduced pressure and allowed to stand for twenty-four hours. At the end of this time it was filtered and as much water as possible removed by distillation in vacuo. The residue was dissolved in absolute alcohol and crystallized by the addition of absolute ether. The material was dissolved in a small quantity of hot water and an equal weight of trinitrophenol (picric acid) added to the boiling solution was shaken with norit [9a] until water clear, then concentrated to a thick syrup in vacuo, dissolved in a minimum quantity of absolute alcohol and crystallized by the addition of absolute ether. The proline was recrystallized from alcohol and ether.

Proline from these various sources gave the same results when used in synthetic mediums. Oxyproline was prepared by the copper salt method of Rose.[7]

It was necessary to adopt a special technic for the preparation of these synthetic mediums. The amino-acids, inorganic salts and dextrose were accurately weighed out and dissolved in distilled water with the aid of heat. It was necessary to get the material into solution at a slightly acid reaction, for it was found that the application of heat to the mediums in alkaline solution resulted in the formation of a flocculent precipitate, which was presumed to be due to the condensation of the amino-acids present to the relatively insoluble di-ketopiperazine derivatives. After cooling to room temperature, the mediums were adjusted to p_H 7.4 to 7.6. The solutions were sterilized by filtration through a Seitz-Werke filter and distributed immediately in sterile test tubes in quantities of 6 cc. The tubed mediums were held at room temperature for five days and incubated at 37 C. for five days. Tubes showing contamination at the end of this time were discarded. The remainder were placed in a jar and the whole evacuated to a pressure of from 15 to 20 mm. of mercury and held there for from fifteen to twenty minutes. At the end of this time, nitrogen was run into the jar and allowed to remain for from two to four hours. Dissolved air is removed in this way to obviate the application of heat to the alkaline solutions, and the introduction of nitrogen precludes the possibility of

7. Personal communication.

8. Biochem. J. **22**:1083, 1928.

9. Biochem. J. **12**:290, 1918.

9a. A brand of beech-wood charcoal.

absorption of atmospheric oxygen before sealing can take place. After removal, the tubes were sealed with a sterile mixture of equal parts of paraffin and petrolatum. The mediums were held at room temperature for five days and incubated at 37 C. for five days. These periods of incubation were judged sufficient to rule out contamination. With careful technic, mediums may be prepared in this way with practically no contamination.

Estimations of growth were made by hemocytometer count, and the identity of the growing organisms was controlled by the demonstration of specific toxin by injection of the culture into unprotected mice and mice protected with antitoxin. When toxin was not found, it was necessary to resort to dilution of the culture sufficient to rule out the survival of inoculated organisms, followed by culture in deep tubes of beef infusion dextrose agar. From this shake culture two colonies were picked and cultured in beef heart medium, and the specific toxigenicity of these cultures was demonstrated by injection into animals.

The presence of toxin was determined by the injection of 0.1 cc. of the culture into white mice weighing from 20 to 25 Gm.

Cultures in synthetic mediums were observed three times at six day intervals. Record was made, at each observation, of turbidity, gas formation, hemocytometer count and the presence or absence of toxin. Negative cultures were kept under observation for a period of two months. Incubation was at 37 C.

EXPERIMENTS

An amino-acid mixture was prepared in which the composition of casein hydrolysate was duplicated, as nearly as possible, at the same time taking into consideration the results of experiments on hydrolysate preparations given in a previous paper.[1] In this first experiment, lysine was lacking, not being available at the time; iron and phosphate were present in traces. Following inoculation with 0.01 cc. of a beef heart culture of strain 3, a scanty growth appeared at the end of four weeks' incubation. This experiment was repeated with lysine added to the medium. In the second case, a suspension of washed spores of strain 3 was used as an inoculum. A small amount of growth appeared at the end of ten days' incubation. The amino-acid composition of this medium is given in table 1 under the heading "Original Mixture."

Experiments were carried out in which this mixture was used with various combinations of inorganic salts. The mediums were made up in (1) Ringer's solution, (2) a solution containing sodium sulphate 0.05 per cent, dibasic potassium phosphate 0.02 per cent and magnesium sulphate 0.0005 per cent, and (3) a solution of potassium and ammonium phosphates, each 0.05 per cent. Growth was decidedly better in the phosphate solution, and consequently the mediums used

TABLE 1.—*Composition of Synthetic Mediums*

Amino Acids	Original Mixture	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6	Medium 7	Medium 8	Medium 9	Medium 10	Medium 11
Lysine	0.1302*	0	0.1313	0.1765	0.1555	0.1494	0.1394	0.1391	0.1461	0.1369	0.1353	0.1314
Glycine	0.0075	0.0085	0	0.0101	0.0089	0.0085	0.0080	0.0080	0.0084	0.0078	0.0077	0.0075
Valine (dl)	0.2614	0.3006	0.2634	0	0.3119	0.2995	0.2795	0.2790	0.2932	0.2744	0.2714	0.2636
Leucine	0.1617	0.1860	0.1629	0.2190	0	0.1853	0.1729	0.1725	0.1814	0.1697	0.1679	0.1631
Proline	0.1273	0.1463	0.1281	0.1722	0.1517	0	0.1360	0.1357	0.1426	0.1335	0.1320	0.1282
Phenylalanine	0.0646	0.0744	0.0651	0.0876	0.0771	0.0741	0	0.0690	0.0725	0.0679	0.0671	0.0652
Glutamic acid	0.0461	0.0531	0.0465	0.0625	0.0551	0.0529	0.0493	0	0.0518	0.0484	0.0479	0.0465
Tyrosine	0.1083	0.1241	0.1092	0.1467	0.1293	0.1241	0.1158	0.1156	0	0.1137	0.1125	0.1091
Histidine	0.0473	0.0544	0.0477	0.0641	0.0514	0.0542	0.0506	0.0505	0.0531	0	0.0491	0.0477
Tryptophan	0.0366	0.0421	0.0399	0.0496	0.0437	0.0420	0.0392	0.0391	0.0411	0.0385	0	0.0369
Cystine	0.0083	0.0095	0.0099	0.0112	0.0099	0.0095	0.0089	0.0088	0.0093	0.0087	0.0086	0

* Throughout the table the figures represent percentages.

in all other experiments were made up to contain, in addition to amino-acids and 0.2 per cent dextrose, 0.05 per cent each of acid potassium phosphate and basic ammonium phosphate. Mediums made up in this way are acid in reaction and may be heated to get the amino-acids into solution, and, after cooling, the hydrogen ion concentration is adjusted with sodium hydroxide.

A series of "elimination" experiments were undertaken to determine, if possible, the relationships of individual amino-acids to the growth of strains 1 and 3. The composition of the mediums used are given in table 1. It was thought desirable to keep the concentration of aminonitrogen and the quantitative relationship between individual amino-acids approximately the same. Consequently, the values were

TABLE 2.—*Growth of Cl. Botulinum on Original Mixture Lacking Tyrosine*

	6 Days				12 Days				18 Days			
	Turbidity	Gas	Count*	Toxin†	Turbidity	Gas	Count	Toxin	Turbidity	Gas	Count	Toxin
Culture 1												
a	+++	+	48.8	0	+++	+	46.5	0	+++	0	45.2	0
b	0	0	0	0	0	0	0	0	0	0	0	0
c	+	0	20.3	0	+	+	26.2	0	++	+	35.6	0
d	+++	0	55.3	0	++++	+	63.9	0	++++	0	56.7	0
e	+	0	18.4	0	+	0	23.9	0	++	+	30.1	0
f	+++	0	48.2	0	+++	+	46.3	0	+++	0	47.9	0
Culture 3												
a	++++	+	56.2	18	++++	+	51.2	12	++++	0	60.1	12
b	++	0	38.7	36	++++	+	49.8	18	++++	+	53.4	18
c	++++	+	61.4	12	++++	+	58.4	12	++++	+	48.7	12
d	0	0	0	0	0	0	0	0	0	0	0	0
e	+	0	24.6	0	++++	+	47.6	24	++++	0	52.5	24
f	0	0	0	0	0	0	0	0	0	0	0	0

* The count is recorded in millions.

† The figures given represent the number of hours elapsing between injection of the culture and death of the animal.

recalculated so that each medium was identical with the original mixture, except that in each case one amino-acid was lacking and the total amounts of the other amino-acids were proportionately increased. Washed spore suspensions were used for inoculation. The results indicated very definitely that growth did not take place in the absence of cystine, proline or leucine, and that the appearance of growth was seriously delayed in the absence of either glycine or lysine. This delayed growth was quite scanty as compared with the relatively abundant growth occurring when these amino-acids were present. A striking feature of the experiments was the superior amount of growth in the absence of tyrosine. This might have been due to adsorption of necessary nutritive materials on tyrosine crystallizing out of solution. Representative protocols are given in tables 2 and 3.

In view of the possible interchangeability of the amino-acids present in these complex mixtures, further experiments were conducted in which the mediums were simplified gradually. The compositions of the mediums used in these experiments are given in table 4. Because of the atoxicity of strain 1 in the experiments described, strain 2, another type B, was used in these experiments. Washed spores were used for inoculation. Abundant growth appeared in mediums 12, 13, 14 and 16 by the fourth or fifth day of incubation. A scanty growth, the appearance of which was delayed ten days took place on medium 18. Mediums 15, 17, 19 and 20 did not show growth. It will be noted that in some of these mediums the deficiency resulting from the removal

TABLE 3.—*Growth of Cl. Botulinum on Original Mixture Lacking Phenylalanine*

	6 Days				12 Days				18 Days			
	Turbidity	Gas	Count*	Toxin†	Turbidity	Gas	Count	Toxin	Turbidity	Gas	Count	Toxin
Culture 1												
a.....	0	0	0	0	+	0	15.3	0	++	+	28.7	0
b.....	+	0	22.4	0	+	+	27.1	0	++	+	32.0	0
c.....	0	0	0	0	0	0	0	0	0	0	0	0
d.....	0	0	0	0	+	0	18.4	0	++	+	34.8	0
e.....	0	0	0	0	0	0	0	0	0	0	0	0
f.....	+	+	18.4	0	++	+	29.2	0	++	0	37.3	0
Culture 3												
a.....	++	+	31.7	18	++	+	33.9	18	++	0	34.0	24
b.....	+	0	22.3	18	++	+	37.5	12	++	+	41.6	24
c.....	0	0	0	0	++	+	28.8	24	++	+	35.4	0
d.....	0	0	0	0	0	0	0	0	0	0	0	0
e.....	++	+	34.7	18	++	+	32.9	12	+++	+	44.3	18
f.....	++	+	27.6	18	++	+	34.5	18	++	0	39.2	12

* The count is recorded in millions.

† The figures given represent the number of hours elapsing between injection of the culture and death of the animal.

of a number of amino-acids was made up by the addition of synthetic alanine, an amino-acid not previously used. It was felt that the delayed growth in the case of medium 18 was due to the absence of lysine. The negative results with medium 20 appear to indicate either that glycine will not serve to take the place of alanine as an indifferent source of nitrogen, or that glycine in such large amounts is toxic. The negative results obtained with mediums 15, 17 and 19 were interpreted to mean that some of the amino-acids were present in sufficiently large quantities to be toxic. Table 5 shows the results of observations on the growth of strains 2 and 3 in medium 16, the simplest medium devised in these experiments.

Further experiments were conducted on mediums 23, 24, 25, 26 and 27, the compositions of which are given in table 4. These experiments were analogous to the "elimination" experiments described, except that medium 16 was used as the starting point instead of the

TABLE 4.—*Composition of Synthetic Mediums*

Amino-Acids	Medium																
	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Proline	0.1273*	0.2337	0.1273	0.2703	0.1273	0.2915	0.1273	0.4190	0.1273	0.1273	0	0	0.1284	0.1610	0.1284	0.1467	0.1273
Glycine	0.0075	0.0140	0.0075	0.0159	0.0075	0.0168	0.0075	0.0246	0.5725	0.0075	0.0075	0.0095	0	0.0095	0.0079	0.0089	0.0075
Leucine	0.1617	0.3070	0.1617	0.3408	0.1617	0.3490	0.1617	0.5310	0.1617	0.1617	0.1617	0.1851	0.1681	0	0.1630	0.1860	0
Isoleucine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1617
Cystine	0.0083	0.0155	0.0083	0.0175	0.0083	0.0191	0.0083	0.0274	0.0083	0.0083	0.0083	0.0095	0.0084	0.0101	0	0.0096	0.0083
Lysine	0.1303	0.2500	0.1303	0.2730	0.1303	0.3000	0	0	0.1303	0.1303	0.1303	0.1478	0.1328	0.1593	0.1315	0	0.1303
Oxyproline	0	0	0	0	0	0	0	0	0	0	0.1273	0	0	0	0	0	0
Tryptophan	0.0366	0.0685	0.0366	0.0725	0	0	0	0	0	0	0	0	0	0	0	0	0
Phenylalanine	0.0649	0.1210	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Histidine	0.0470	0.1012	0	0	0	0	0	0	0	0.0470	0	0	0	0	0	0	0
Alanine (dl)	0.4630	0	0.5280	0	0.5647	0	0.6951	0	0	0.5176	0.5647	0.6460	0.5740	0.7910	0.5670	0.6480	0.5647

* Throughout the table the figures represent percentages.

original mixture. Delayed and scanty growth was encountered in mediums 24 and 27, and the other mediums showed no growth. It was noted that mediums in which no growth appears do not become toxic from standing after inoculation—at least not to a degree detectable by the technic used.

Further experiments were conducted in an effort to substitute oxyproline for proline and isoleucine for leucine in medium 16. Mediums 22 and 28 were used. In both cases the substitution was successful.

Throughout these experiments growth was somewhat irregular. This was taken care of by the use of at least six cultures of each

TABLE 5.—*Growth of Cl. Botulinum on Medium 16, Containing Only Six Amino-Acids*

	6 Days				12 Days				18 Days			
	Turbidity	Gas	Count*	Toxin†	Turbidity	Gas	Count	Toxin	Turbidity	Gas	Count	Toxin
Culture 2												
a	0	0	0	0	0	0	0	0	0	0	0	0
b	0	0	0	0	0	0	0	0	0	0	0	0
c	++++	+	67.1	0	++++	+	72.3	0	++++	0	59.8	0
d	0	0	0	0	0	0	0	0	0	0	0	0
e	++++	+	61.0	0	++++	+	68.7	0	++++	0	47.5	0
f	0	0	0	0	0	0	0	0	0	0	0	0
Culture 3												
a	0	0	0	0	++++	+	65.4	0	++++	+	63.2	36
b	+++	+	58.3	0	++++	+	73.5	12	++++	0	68.5	12
c	+++	+	65.2	0	++++	+	74.1	12	++++	0	57.9	18
d	0	0	0	0	0	0	0	0	0	0	0	0
e	+++	0	48.7	0	++++	+	58.2	72	++++	+	65.4	18
f	0	0	0	0	0	0	0	0	0	0	0	0

* The count is recorded in millions.

† The figures given represent the number of hours elapsing between injection of the culture and death of the animal.

organism used in any given medium. This irregularity may be accounted for in two ways. First, the irregularity of germination of spores is well known. Second, it was found that all mediums showing growth gave a positive nitroprusside test, while those showing no growth did not. This indicated that a sufficiently low reduction potential had not been attained. It was found that irregularities of growth may be smoothed out to a considerable extent by the use of washed vegetative cells suspended in a solution of cysteine hydrochloride as inoculums. However, some irregularity still remains, and at times a batch of medium, apparently identical with other batches which support growth, proves to be entirely unsatisfactory. The reason for this is unknown.

Throughout these experiments no toxin was found in type B cultures in synthetic mediums. Type A toxin was demonstrated with

fair regularity. It was noted that in the absence of tryptophan toxin was not detectable after six days' incubation even when profuse growth had taken place, but was found after twelve days' incubation. When tryptophan was present, toxin was detectable at six days. Titrations, carried out by dilution in saline, indicated that toxin was present to the extent of 100 M. L. D. per cubic centimeter either with or without tryptophan and did not increase with continued incubation. It was noted also that the toxin formed acted very quickly—0.01 cc., injected subcutaneously, killed mice weighing from 20 to 25 Gm. in from two to four hours. None of the usual symptoms of botulinum intoxication was apparent until from ten to fifteen minutes before death. Mice protected with antitoxin did not succumb.

Three attempts were made at different times to grow the anaerobes Cl. welchii, Cl. tetani, Cl. chauvaei, Cl. histolyticum and Cl. sporogenes in medium 16. None of these attempts was successful.

Studies of growth in transfer in these synthetic mediums are difficult because of irregularities of growth. It was possible, however, to transfer strain 3 three times in the original mixture, but the organisms failed to grow on the fourth transfer.

COMMENT

The experimental results presented here seem to support the hypothesis of essential amino-acids for these cultures of Cl. botulinum. The weakness of the hypothesis lies in the fact that its verification depends on negative results. At present, it cannot be said with certainty that the failure to grow was due entirely to variation in the nutritive materials of the culture medium, for it is quite possible that unknown and uncontrolled variables might have brought about this effect. For example, the reduction potential was not accurately controlled in these experiments. It is felt, however, that the positive results recorded here have some value.

Modification of these mediums by the introduction of unknown materials was kept at the lowest possible level by the use of washed spores and washed vegetative cells for inoculation. A slight degree of modification, however, cannot be entirely eliminated, for the inoculated organisms constitute contamination in a chemical sense.

The failure to grow other obligate anaerobes in medium 16, while not conclusive by any means, suggests that the medium is specific for the strains of Cl. botulinum used. It may be noted here that the usual spore-forming contaminants grew readily in all these mediums.

It is of considerable interest that tryptophan is not necessary for growth. Further observations, however, on growth in casein and gelatin hydrolysate mediums, as yet unpublished, have indicated that

the function of tryptophan in promoting growth in casein hydrolysate mediums may be partially taken over by cystine. Conversely, the function of sulphydryl compounds and cysteine, in particular, in promoting growth on gelatin digest mediums as reported by Hosoya and Kishino [10] and by Quastel and Stephenson [11] may be taken over by tryptophan. This, however, did not prove to be true in these synthetic mediums. The relation of tryptophan to anaerobiosis is discussed by Braun and Cahn-Bronner.[12]

Complex mixtures of inorganic ions were apparently not necessary to the growth of these organisms. This is not interpreted to mean that such ions are not necessary to growing protoplasm, but rather that only such minute quantities as are present as contamination are necessary. It will be recalled that the amount of iron necessary for oxidation catalysis in Warburg's sense is in the neighborhood of 1 : 50,000,000 mols.[13]

The formation of botulinum toxin in these mediums is deserving of comment. No explanation is offered for the fact that while type A toxin was demonstrated with fair regularity, no type B toxin was found at any time. The type B cultures used were toxigenic in ordinary beef heart medium.

SUMMARY

Evidence supporting the hypothesis of essential amino-acids for bacteria has been presented.

Certain strains of Cl. botulinum grew well on certain synthetic mediums.

The amino-acids cystine, leucine and proline were essential to the growth of the strains of Cl. botulinum used.

The amino-acids lysine and glycine, while not essential, were active in promoting growth of these strains of Cl. botulinum.

Isoleucine and oxyproline could be substituted for leucine and proline respectively as essential amino-acids.

The obligate anaerobes, Cl. welchii, Cl. chauvaei, Cl. sporogenes, Cl. histolyticum and Cl. tetani would not grow on a simple synthetic medium which would support the growth of these strains of Cl. botulinum.

The type A culture of Cl. botulinum used showed better growth on these mediums than either of the two type B cultures.

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11. Biochem, J. **20**:1126, 1926.

12. Centralbl. f. Bakt. (Abt. 1) **86**:196 and 380, 1921.

13. Meyerhof, O.: Chemical Dynamics of Life Phenomena, Philadelphia, J. B. Lippincott Company, 1924.